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CHEMICAL AND BIOLOGICAL STRATEGIES FOR ENGINEERING CELL SURFACE GLYCOSYLATION

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■ **Abstract** Oligosaccharides play a crucial role in many of the recognition, signaling, and adhesion events that take place at the surface of cells. Abnormalities in the synthesis or presentation of these carbohydrates can lead to misfolded and inactive proteins, as well as to several debilitating disease states. However, their diverse structures, which are the key to their function, have hampered studies by biologists and chemists alike. This review presents an overview of techniques for examining and manipulating cell surface oligosaccharides through genetic, enzymatic, and chemical strategies.

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INTRODUCTION

Over the past three decades the prevailing attitude toward cellular glycosylation has evolved (Cook 1995). Once viewed as a hydrophilic heterogeneous nuisance to be removed from cell surface or secreted proteins of interest, oligosaccharides are now recognized as an intricate network encoding a wealth of information. One stimulus for this shift in perspective is the recent development of the necessary tools to study complex, biologically relevant carbohydrates. Unlike amino acid and nucleic acid polymers, carbohydrate chains are not typically linear, they are not biosynthesized from a template, and until very recently (Plante et al. 2001, Koeller & Wong 2000) they could not be chemically synthesized using programmable instruments. This precludes the use of many straightforward genetic techniques and mandates chemical synthesis in order to obtain the compounds of interest in homogeneous form and in sufficient quantity. It is the challenge of deciphering the purposes of glycosylation that now draws many researchers into the field of glycobiology. As our knowledge of glycoconjugate biosynthesis has increased, so has our ability to control and manipulate glycosylation patterns. This, in turn, has led to new insights into cell biology and the development of new approaches to cellular engineering. Here we provide an overview of techniques for engineering cell surface glycosylation, both through modulating the activity of the enzymes that construct cell surface oligosaccharides and through selective chemical reactions of the carbohydrates themselves.

Biological Significance of Cell Surface Glycosylation

Carbohydrates on the cell surface contribute to most interactions between the cell and its environment (Varki 1993, Dwek 1996). Because they form the outermost layer of the cell, carbohydrates are the first molecules to be encountered and recognized by other cells, (Figure 1*a*), antibodies (Figure 1*b*), invading viruses (Figure 1*c*), and bacteria (Figure 1*d*). Secreted molecules such as hormones and toxins have also been shown to bind carbohydrate receptors on the cell surface (Figure 1*e*). In addition to serving as recognition elements, cell surface carbohydrates play a particularly important role in regulation of the immune response. Most of the receptor proteins that mediate lymphocyte interactions are glycosylated, endowing them with increased protease resistance and allowing them to assume the necessary conformation and cell-surface clustering for proper functioning (Rudd et al. 1999). Given the role of glycosylation in cellular communication, it is not surprising that abnormal glycosylation patterns are known to be markers for, and in some cases the cause of, certain disease states including cancer (Dennis et al. 1999).

The focus of this review is cell surface glycosylation; however, the biological importance of oligosaccharides goes much deeper. The presence of the appropriate oligosaccharide can ensure correct folding of a protein as it traverses the endoplasmic reticulum, without which it would be misfolded and inactive (Wormald &

Dwek 1999, Imperiali & Rickert 1995). Furthermore, glycosylation can act as the key regulatory switch for protein activity. This was clearly demonstrated by the discovery that activity of the Notch receptor is directly modulated by glycosylation by the Fringe glycosyltransferase, ensuring correct development in *Drosophila* (Moloney et al. 2000, Bruckner et al. 2000, Munro & Freeman 2000). The regulation of protein activity via glycosylation also occurs within the nucleus. The processes of translation and transcription are thought to be intricately controlled by reversible glycosylation of transcription factors and RNA polymerase, much in the same way that phosphorylation is known to influence these processes (Comer & Hart 2000, Shafi et al. 2000).

Structural Classification of Glycoforms

As mentioned above, the structures of the oligosaccharides that perform these important functions are elaborate. Unlike other biomolecules, oligosaccharides are diverse with respect to both the number of monomers and the observed linkage patterns (Rudd & Dwek 1997a). There are nine common cell surface monosaccharides that are assembled to give the prototypical structures shown in Figures 2 and 3. These units can be linked to each other at numerous positions around the sugar ring and in two distinct geometries (α and β) at the glycosidic linkage. Glycoconjugates can be classified into five categories based on the five main scaffolds on which their oligosaccharides are bound: *O*-linked glycoproteins, glycosaminoglycans, *N*-linked glycoproteins, GPI-anchored proteins, and glycolipids.

O-linked glycosylation (Figure 2*a*) is initiated by the attachment of a single monosaccharide, usually *N*-acetylgalactosamine (GalNAc), to a serine or threonine residue within a protein (VandenSteen et al. 1998). This proximal sugar is then further elaborated by a series of glycosyltransferases to form the mature *O*-glycan structure; one example of which is the sulfated core 2 glycan (Figure 2*a*), a key ligand involved in the process of leukocyte extravasation (Rosen & Bertozzi 1996). Glycosaminoglycans are linear-repeating polymers that include heparan sulfate, chondroitin sulfate, keratan sulfate, and dermatan sulfate. They all share a conserved core tetrasaccharide bound to serine or threonine residues (Figure 2*b*). *N*-linked glycosylation takes place with the transfer of a 14-residue oligosaccharide, rather than a single sugar, to a nascent polypeptide at the sequence Asn-X-Ser/Thr, where X is any amino acid excluding Pro. This oligosaccharide undergoes enzymatic trimming to form a core structure, the simplest of which is shown in Figure 2*c*. Diversification occurs via the action of several glycosyltransferases that append additional sugar units to the terminal mannose residues (Kornfeld & Kornfeld 1985).

In contrast to these two classes of glycoproteins, glycosylphosphatidylinositol-(GPI) anchored proteins possess oligosaccharides as part of the structure linking them to the cell membrane, rather than at the external fringe (Nosjean et al. 1997). This structure, unlike *N*- and *O*-glycans, possesses little variability in the

monosaccharide sequence (Figure 3*a*). Glycolipids also present oligosaccharides proximal to the cell membrane and are often found associated with cell surface receptors. For example, GM_3 (Figure 3*b*) has been shown to modulate the activity of the EGF receptor and, therefore, cell growth (Rebbaa et al. 1996).

Challenges to the Study and Engineering of Physiological and Pathological Glycosylation

Considering that oligosaccharide monomers are of extremely similar chemical reactivity and that the polymers they make up are heterogeneous both in sequence and spatial arrangement, discerning the structure of different oligosaccharides is not an easy task. In a typical structure determination experiment, the sugars are released from the protein using either chemical or enzymatic methods, and then a series of techniques for labeling (e.g., radioisotopes, fluorescent tags), separation (e.g., HPLC, SDS PAGE, high pH anion exchange chromatography), and analysis (e.g., mass spectroscopy, matrix-assisted laser desorption ionization) are needed to characterize the glycans (Rudd & Dwek 1997b, Venkataraman et al. 1999). Oligosaccharide heterogeneity presents a challenge not only in deciphering a structure of interest, but also in identifying the multiple enzymes that are involved in its biosynthesis. Each class of glycoconjugate requires different enzymes for its assembly and a specific enzyme (or family of enzymes) for each type of glycosidic linkage. The multiple genes encoding these enzymes must be expressed in concert and then localized to the Golgi apparatus where the process of glycosylation is sequestered within the cell. With these factors in mind, it becomes evident that observing the activity of an isolated glycosyltransferase in vitro cannot begin to approximate the native context in which it normally functions. In order to understand and control the activity of the enzymes that synthesize oligosaccharides and to elucidate the function of the carbohydrates themselves, these components must be studied in the context of the cell. The remainder of this review describes the development of methods for studying, and ultimately, for specifically altering glycoconjugates in a cellular environment.

ALTERING GLYCOSYLATION AT THE GENETIC LEVEL

Glycosyltransferase Mutants

Enzymes that synthesize carbohydrate structures by transferring specific monosaccharide units from a sugar nucleotide onto a growing oligosaccharide chain are known as glycosyltransferases. Many important insights about the carbohydrate product of a glycosyltransferase can be obtained by studying cell lines in which that enzyme has been either introduced or deleted at the genetic level (Stanley & Ioffe 1995). Cell lines can be engineered to express a glycosyltransferase that was otherwise absent or expressed at significantly lower levels in wild-type cells. These

engineered cells can now synthesize carbohydrate epitopes of interest, allowing the biosynthesis and function to be observed in the context of the cell. Alternately, a cell line in which a specific glycosyltransferase has been removed allows processes that are dependent upon the carbohydrate product of that enzyme to be identified because they will have been disrupted. Such genetically engineered glycosylation mutants provide an arena in which to study the molecular basis of carbohydrate function.

The earliest work with mammalian glycosylation mutants made use of a Chinese hamster ovary (CHO) cell line lacking an *N*-acetylglucosaminyltransferase (GlcNAcT) activity (Stanley et al. 1996a). Removing the enzyme, now called GlcNAcT-I, resulted in the discovery of the structure of its biologically relevant substrate. These studies are extremely important in matching each enzyme with its appropriate targets and functions. The existence of GlcNAcT-II was also revealed in this mutant cell line. It is now known that there are multiple GlcNAcTs in humans, some of which appear to be redundant and others whose biological product is unknown.

Cell lines that have gained rather than lost glycosyltransferase activity are equally informative. *N*-linked glycans with unique carbohydrate structures were discovered in mutant CHO cells that had undergone de novo expression of a novel GlcNAcT (Stanley et al. 1996b). Such a phenotype can be engineered by transfection of cells with a suitable GlcNAcT. The resulting exotic carbohydrate epitopes alter the cell's interactions with antibodies and other cells. For example, transfection of the EL-4 T lymphoma cell line with core 2 GlcNAcT resulted in altered glycoforms on the CD43 polypeptide and subsequent changes in antibody binding (Barran et al. 1997). Similar shifts in glycosylation patterns have been associated with certain immune disorders such as rheumatoid arthritis and AIDS. Transfection of the same enzyme into T cells generated an epitope recognized by Galectin-1 that resulted in apoptosis. This suggests that core 2 GlcNAcT may be involved in regulation of the immune system by instigating T cell death (Galvan et al. 2000).

The functions of the family of *N*-acetylgalactosaminyltransferases (GalNAcTs) are no less complex. GalNAc is a particularly interesting monosaccharide residue because it initiates *O*-linked glycosylation in vertebrates. Currently, eight human GalNAcTs have been identified and cloned. Selective disruption of these genes is beginning to shed light on their different roles in glycosylation (Marth 1996). The GlcNAc and GalNAcTs are enzymes that install monosaccharides at sites close to the protein and appear to be important for modulating protein conformation and activity. On the other hand, enzymes that append terminal sugars on oligosaccharides have the most impact on cell-cell adhesion interactions. Fucose and sialic acid are common terminal sugars, and the enzymes that install them, fucosyltransferases (FucTs) and sialyltransferases (SiaTs), have been shown to play important roles in leukocyte adhesion processes. Both enzymes are required for the synthesis of sialyl Lewis x (sLe^x, Figure 2*a*), the key carbohydrate epitope that mediates binding of leukocytes to the activated endothelial cells lining the walls of blood vessels at sites of inflammation. This initial interaction is followed by extravasation of the leukocyte into injured tissue. Studies in which FucTs or SiaTs were transfected

into heterologous cell lines were instrumental in defining the roles of fucose and sialic acid in inflammation (Lowe et al. 1990, Patnaik et al. 2000).

Carbohydrates can serve as antigenic determinants, a fact that was established long ago with the molecular characterization of the human blood type oligosaccharides. The antigenic properties of foreign oligosaccharides promote a vigorous immune response against invading organisms. The structure Galα1-3Galβ1-4GlcNAc (αGal), for example, is not produced by humans but is prevalent in microbes; exposure to these foreign antigens has stimulated humans to produce large quantities of anti-αGal IgG, as much as 1% of total circulating IgG. The strength of this response has hampered recent efforts to use porcine organs, which express αGal, as a source of transplant tissue for humans. One strategy to circumvent this problem has involved transfecting swine endothelial cells with other glycosyltransferases that compete for the same substrate as α1,3-galactosyltransferase (Koma et al. 2000).

Glycosidase Mutants

Whereas the glycosyltransferases add sugars onto proteins, lipids, or other carbohydrates, the glycosidases are charged with the responsibility of degradation. Glycosidases are enzymes that cleave sugars from glycoconjugates, e.g., the sialidases (neuraminidases), enzymes that removes sialic acid from cell surface glycoconjugates. Given that sialic acid residues are determinants of certain processes critical for the immune response, it follows that a sialidase activity would be necessary in order to regulate such processes (Bagriaaik & Miller 1999). Possibly the most studied sialidase is the viral sialidase, which plays an important role in influenza infectivity. The influenza virus binds to sialic acid residues on the cell that it invades and later cleaves those sialic acids with a viral neuraminidase, thereby allowing the release of the viral progeny (Kiefel & von Itzstein 1999). The requirement for neuraminidase activity was demonstrated using a mutant virus lacking this enzyme. The mutant virus was able to enter cells and replicate similarly to wild-type virus but was unable to elute from the host cell (Liu & Air 1993). Based on such observations, inhibitors of neuraminidase have been developed, some of which have proven to be clinically effective against influenza (Lew et al. 2000).

Transgenic Mice as Models of Glycoconjugate Function

Most cell surface oligosaccharides regulate multicellular processes. Therefore, studies that address their function within an organism rather than on isolated cells in culture often provide a more complete picture of cell surface carbohydrate function. Transgenic expression of glycosyltransferases in mice has been a powerful tool for elucidating their roles in metabolism, development and disease (Marth 1994, Hennet & Ellies 1999, Manzi et al. 2000). This technology has been particularly useful in deciphering the contribution of the different FucTs and SiaTs to leukocyte adhesion (Hennet et al. 1998, Maly et al. 1996, Weninger et al. 2000, Huang et al. 2000). Mouse models have also been instrumental in attempts to understand the

human response to the αGal antigen. Mice deficient in α1,3-galactosyltransferase have been used as models for humans who naturally lack this enzyme (Thall et al. 1996). These model organisms were used to investigate how the immune response to αGal may be minimized, potentially allowing successful xenotransplantation (Tanemura et al. 2000).

The disadvantage to studying glycosyltransferases in vivo is that the phenotype caused by a genetic mutation is not always evident or interpretable. Frequently a mutation will result in an embryonic lethal phenotype, indicating that the product of that gene is necessary for the organism's development but preventing studies on the role of the enzyme in post-developmental processes. In addition, the mutation may produce no discernable abnormal phenotype, which occurs when other glycosyltransferases can compensate for the lost activity. These problems are being addressed by advanced knockout techniques that are either inducible or tissue specific.

MODULATING GLYCOSYLTRANSFERASE AND GLYCOSIDASE ACTIVITY WITH SMALL MOLECULES

Abrogation of Cell Surface Glycosylation

Small molecule inhibition of carbohydrate-modifying enzymes has proven to be a successful method of rapidly and selectively interfering with cell surface glycosylation. The use of either naturally derived or synthetic inhibitors is also more widely applicable than selection of mutant cell lines because the small molecule can be applied to tissues of any organism, irrespective of its genetic tractability. These inhibitors can be natural products, designed substrate mimics, or glycoside primers.

The most widely used inhibitor of *N*-linked glycosylation is tunicamycin, a natural product first identified in *Streptomyces lysosuperificus*. Tunicamycin (see Figure 4*a*) eliminates all *N*-linked glycosylation in eukaryotes by inhibiting the first GlcNAcT responsible for the construction of the lipid-linked tetradecasaccharide donor for *N*-linked glycosylation. This tetradecasaccharide is transferred onto a target protein by the enzyme oligosaccharyl transferase (OT). The amount of tunicamycin required to inhibit glycosylation is cell line–dependent; however, doses of 0.1–10 μ/mL of cell culture media are usually sufficient to prevent *N*-glycan formation without killing the cells, a frequent problem at higher doses (Elbein 1987). Tunicamycin acts early in the process of *N*-linked glycan construction. Thus exposure to tunicamycin prevents the presentation not simply of a single monosaccharide but rather of all monosaccharides that normally reside within *N*-linked glycans. It has, therefore, been most useful for determining the contribution of *N*-glycans to a particular cellular process and determining whether a carbohydrate of interest is an *N*-linked carbohydrate.

Another avenue for inhibiting the assembly of *N*-linked glycans is to inhibit OT. Imperiali and coworkers designed inhibitors of OT based on the consensus

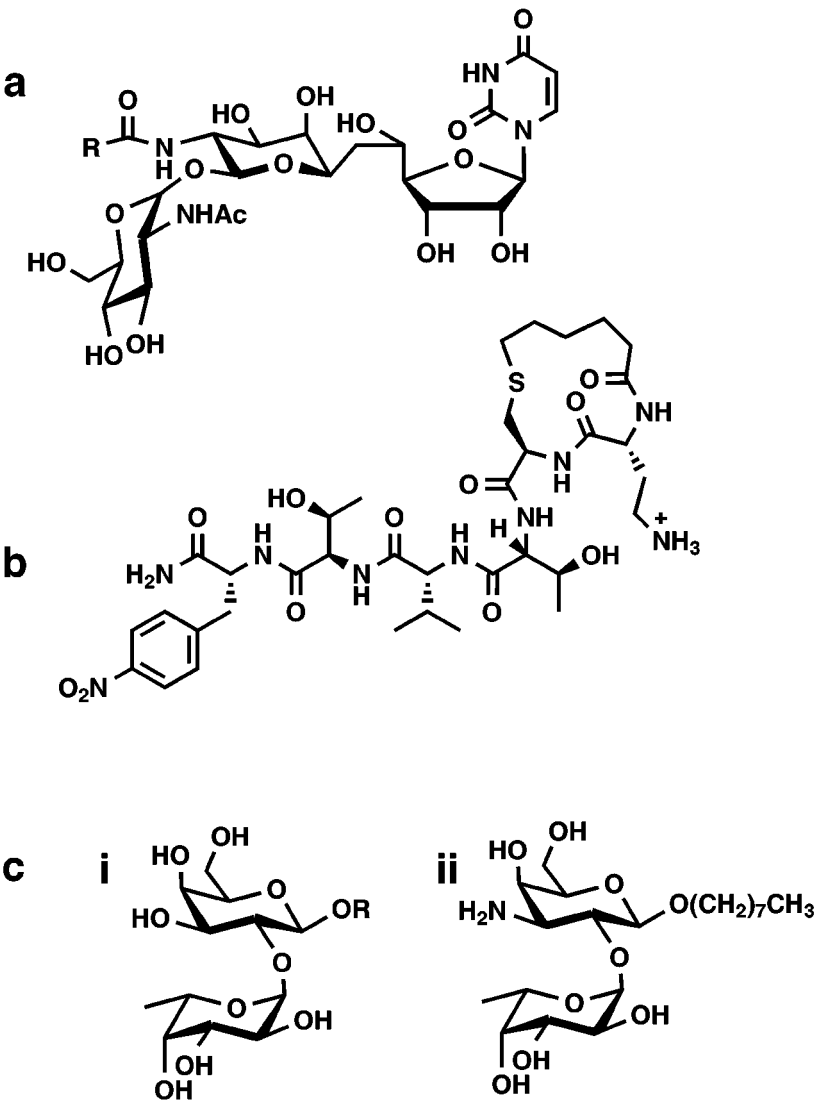


Figure 4 Small molecule inhibitors of cell surface glycosylation: (*a*) tunicamycin, a natural product found to inhibit *N*-linked glycosylation; (*b*) a cyclic peptide inhibitor of oligosaccharyl transferase; (*c*) **i**, precursor to the blood group A trisaccharide and the substrate for a GalNAcT; **ii**, inhibitor designed to mimic the GalNAcT substrate.

sequence, Asn-X-Ser/Thr, of its protein substrates. The enzyme was shown to be inhibited very effectively by a series of peptides related to that shown in Figure 4*b* (Kellenberger et al. 1997). A panel of peptide analogs was synthesized in order to obtain more powerful inhibitors, resulting in K_is as low as 10 nM (Ufret & Imperiali 2000, Eason & Imperiali 1999). The peptidic nature of these inhibitors of OT compromised their bioavailability and, hence, in their present form, they cannot be used to disrupt *N*-linked glycosylation in cell-based systems. However, methods for improving cell permeability of peptide drugs may be applied to this system, thus providing a potentially useful tool for cell biologists.

One strategy for designing inhibitors of glycosyltransferases is to synthesize a close mimic of the native substrate. This has proven successful for selectively inhibiting many glycosyltransferases in vitro including GlcNAcTs (Lu et al. 1997), GalTs (Helland et al. 1995), FucTs (Qiao et al. 1996), and SiaTs (Schaub et al. 2000). An example where this technique has transferred successfully to a cell-based assay is the inhibition of a GalNAcT involved in blood group synthesis (Laferte et al. 2000). The enzyme normally transfers GalNAc from UDP-GalNAc onto the disaccharide substrate **i** (Figure 4*c*) to give the blood group A trisaccharide. The designed inhibitor **ii** (Figure 4*c*) is able to inhibit this transfer with a K_i of 200 nM by effectively mimicking the disaccharide substrate. In order to assay for reduction of blood group A synthesis in a cellular context, a fluorescently labeled derivative of **i** was used. Co-incubation of cells with the fluorescent substrate and the inhibitor resulted in decreased production of the fluorescent trisaccharide product relative to its observed levels in the absence of the inhibitor, as monitored by capillary electrophoresis. Although the labeled trisaccharide product is not delivered to the cell surface, it follows that the inhibitor has the same effect on production of protein-associated blood group A, thereby limiting its presentation on the surface of living human carcinoma cells (Le et al. 1997).

The glycosylation inhibitors discussed thus far act by binding to the enzyme that installs the carbohydrate. Another effective route is to provide those enzymes with an excess of an alternate substrate that serves to divert the enzyme from the intended protein or lipid. This technique was first demonstrated using the designed primer α-benzyl-GalNAc (Figure 5*a*). The primer mimics the structure of the proximal GalNAc on an *O*-linked glycoprotein such as the heavily glycosylated mucins. The compound was shown to decrease mucin glycosylation by over 80% in a variety of different cancer cell lines without affecting growth rate or viability. Benzyl glycosides secreted from these cells were found to be comparable in size to those that are found on the mucins of untreated cells, confirming that α-benzyl-GalNAc is a substrate mimic and not an enzyme inhibitor. A decrease in the abundance of mucin chains using α-benzyl-GalNAc led to a decrease in the metastatic capacity of colon cancer cells in both in vitro and in vivo models (Huang et al. 1992, Byrd et al. 1995). Thus the inhibitor provided insight into the biological significance of mucin glycosylation on tumor cells.

The principle of feeding cells a soluble substrate decoy to modify cell surface glycosylation was extended to include other oligosaccharides. The primer

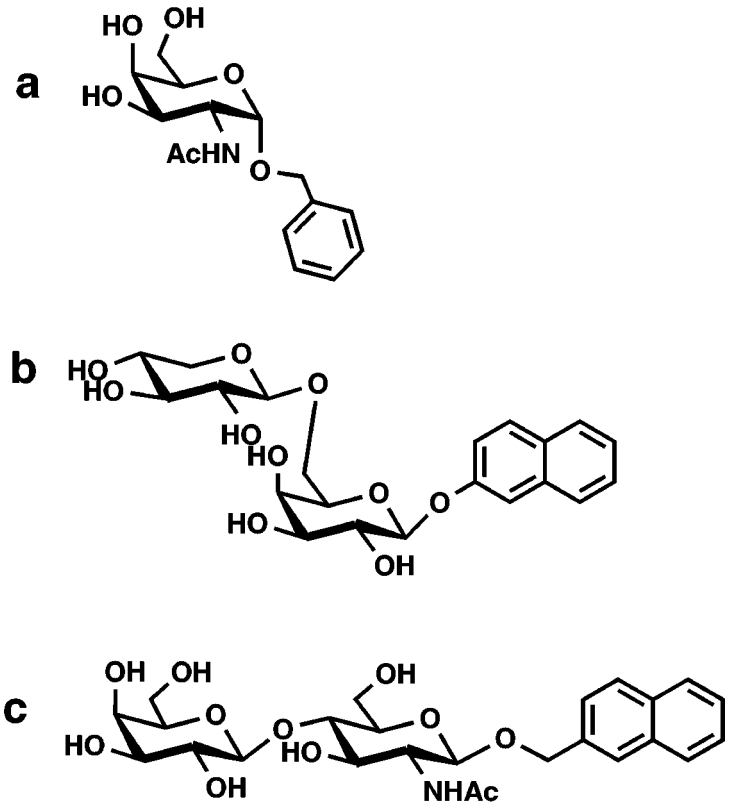


Figure 5 Primers for oligosaccharide synthesis: (*a*) α-benzyl-GalNAc, (*b*) Xylβ-6Galβ-*O*-2-naphthol, (*c*) Galβ1-4GlcNAcβ-*O*-naphthalenemethanol.

Xylβ1-6Galβ-*O*-2-naphthol (Figure 5*b*) was used as a substrate for glycosaminoglycan synthesis in CHO cells, and Galβ1-4GlcNAcβ-*O*-naphthalenemethanol (Figure 5*c*) was found to be a primer for sLe^x synthesis in HL-60 cells (Sarkar et al. 1995). In the latter, the primer was found to be fucosylated in favor of the endogenous sLe^x precursor, which resulted in suppression of cell surface sLe^x and impaired ability to participate in sLe^x-mediated adhesion (Sarkar et al. 1997). In both cases, the primer was a disaccharide that would normally have low membrane permeability relative to a monosaccharide. In order to ensure that the substrates would be taken up by cells, the primers were acetylated. The acetate esters were enzymatically cleaved by esterases within the cell, leaving the unprotected disaccharide available as a substrate for glycosyltransferases.

Treating cells with soluble primers reduces the density of cell surface oligosaccharides and therefore compromises the binding or recognition function that the

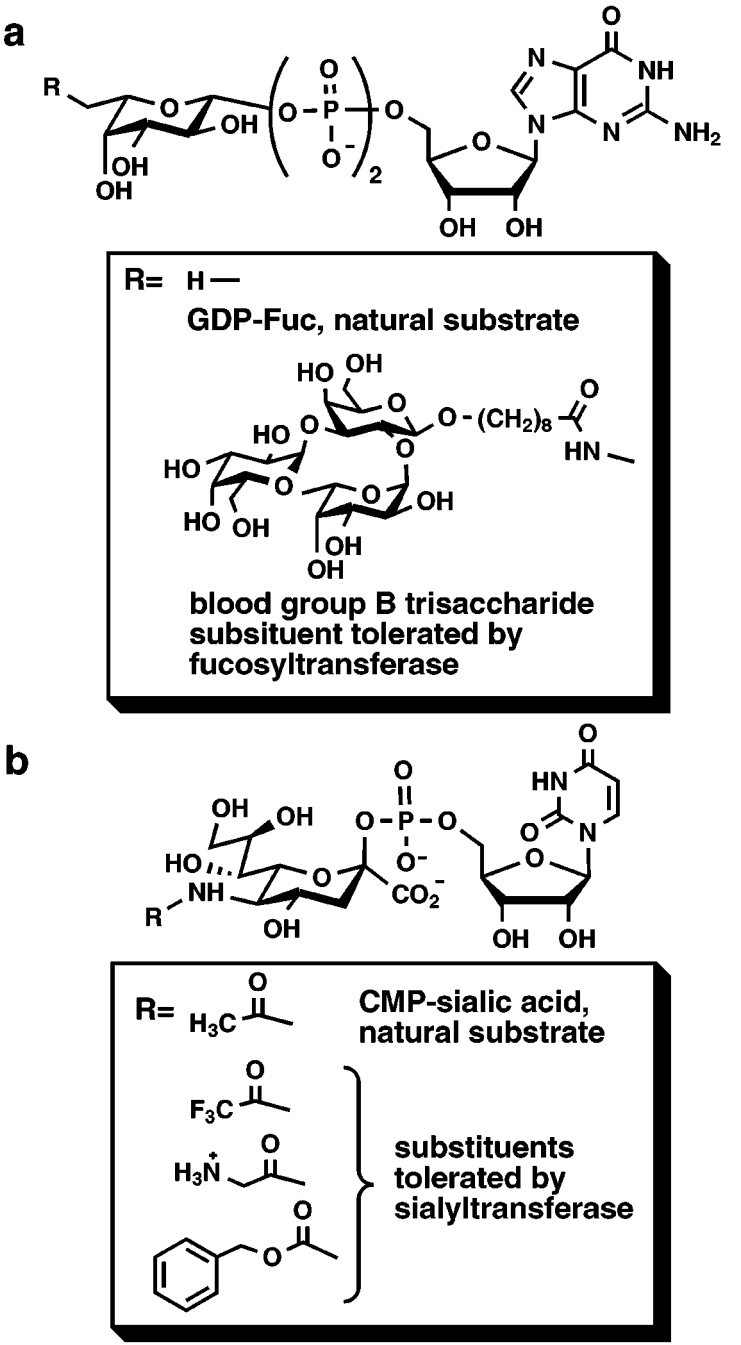
oligosaccharide performs for the cell. However, in some cases, the presence of the oligosaccharide in its soluble aryl glycoside form can restore function. Heparan sulfate proteoglycans (HSPG) are soluble glycoproteins normally associated with the cell surface and are required for the presentation and activity of basic fibroblast growth factor (bFGF). A mutant cell line lacking the glycosyltransferase that initiates HSPG synthesis is unable to bind bFGF. When treated with a xyloside primer that mimics the missing xylose-modified protein, these mutant cells were found to bind bFGF in a manner similar to wild-type cells (Miao et al. 1995).

Creating New Oligosaccharide Structures on Cell Surfaces

Perturbing a normal mode of glycosylation will interrupt processes in which the displaced carbohydrates participate. However, it is also possible to do the reverse experiment and observe what happens when new or even unnatural oligosaccharides are installed on the cell surface, thereby allowing a glycosylation-dependent process to be reconstituted or a novel one to be engineered. Glycosidase inhibitors prevent the trimming of *N*-glycans as they are processed into their final cell surface form. The most effective examples of these inhibitors are a series of plant-derived alkaloids that cause presentation of immature, and therefore unnatural, oligosaccharides on the cell surface. These compounds act as a chemical defense system for the plants that produce them; however, they can also serve as useful tools for studying the biosynthesis of glycoproteins (Elbein 1991).

A powerful technique for creating new oligosaccharide epitopes on cell surfaces involves exogenous delivery of both a glycosyltransferase and its substrate. Recent studies have shown that it is possible to transfer a surprisingly wide array of unnatural carbohydrate molecules onto the cell surface using enzymes with relaxed specificity for the structure of the donor molecule and, in some cases, for the acceptor as well. Certain enzymes, such as the fucosyltransferases, are extremely permissive for large substituents at the C-6 position of fucose in their substrate GDP-fucose (Figure 6*a*). This means that well-characterized synthetic oligosaccharides can be delivered to the cell surface in a controlled fashion (Srivastava et al. 1992, De Vries et al. 1997). Glycosyltransferases are frequently used in the construction of the oligosaccharide of interest because they require no protection and deprotection steps, reaction conditions are mild, and stereo-selectivity is unparalleled (Ernst & Oehrlein 1999).

This has been particularly useful in elucidating the structures responsible for leukocyte adhesion. Early in the inflammatory response, leukocytes and endothelial cells recognize each other through the binding of adhesion molecules, called selectins, to carbohydrate epitopes on the cell surface. E- and P-selectin on endothelial cells bind to carbohydrates on leukocytes, and conversely, L-selectin on leukocytes binds to carbohydrates on endothelial cells. Identification of the structures of the carbohydrate ligands provides critical information for development of inhibitors that block this interaction, which in turn prevent the excessive leukocyte influx that is the hallmark of severe inflammatory disease (Rosen 1999). When


a
R=
H—
GDP-Fuc, natural substrate
blood group B trisaccharide subsituent tolerated by fucosyltransferase
b
R=
CMP-sialic acid, natural substrate
substituents tolerated by sialyltransferase

two potential selectin ligands, one sulfated and the other unsulfated, were attached to lipids and tested for selectin binding, both were found to bind equally well. However, when these same oligosaccharides were transferred onto the cell surface by the promiscuous fucosyltransferase, the two bound very differently to E- and P-selectin (Tsuboi et al. 2000). Similar studies were used to define the importance of the sulfate esters in the oligosaccharide, depicted in Figure 2*a*, to selectin-mediated cell adhesion (Tsuboi et al. 1996).

Sialyltransferases are similarly open to modification of their substrates (Figure 6*b*). Using a number of different sialyltransferases, a panel of sialic acids with modified substituents at the C-5 position were transferred onto human erythrocytes to determine the contribution of each substituent to lectin binding (Gross & Brossmer 1995, Kelm et al. 1998). The fucosyl and sialyltransferases have been utilized extensively to transfer unnatural fucose or sialic acid variants, respectively. However, some unnatural substrates are not recognized by these enzymes. This limitation can be overcome by engineering the target enzymes to alter their specificity, as was demonstrated by converting a blood group A GalNAcT into a blood group B GalT through mutation of just four amino acids (Seto et al. 1999).

As mentioned previously, the αGal epitope is highly antigenic in humans. This property has been exploited for new approaches to tumor immunotherapy. The glycosyltransferase that constructs the αGal epitope has been used to enzymatically transplant αGal onto human tumor cells. Reintroduction of the engineered cells to the patient resulted in a strong and specific immune response to αGal, thus facilitating the degradation of the tumor cells and subsequent antigen presentation (LaTemple et al. 1996, Galili & Anaraki 1995).

METABOLIC INCORPORATION OF UNNATURAL SUBSTRATES

In theory, glycosyltransferases can be engineered and utilized to synthesize any unnatural oligosaccharide of interest. However, there are two major restrictions that prevent this technique from being universally applicable. First, the sugar nucleotide donor must be synthesized, which can be an arduous process. Second, the enzyme itself must be available and in such quantities as to be useful for preparative purposes. A much more efficient procedure is to co-opt the cells own machinery for synthesizing natural cell surface oligosaccharides and use it to generate unnatural structures. This method retains the caveat that the enzymes involved in

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Figure 6 Unnatural substrates utilized by the fucosyl and sialyltransferases: (*a*) GDP-fucose, and an unnatural substrate with the blood group B trisaccharide appended to the C-6 position of GDP-fucose; (*b*) CMP-sialic acid and examples of unnatural analogs tolerated by the sialyltransferases.

the biosynthetic pathway must be able to accommodate the unnatural sugar(s) of interest.

Feeding cells unnatural sugars that are analogs of a metabolic precursor of a cell surface carbohydrate often results in inhibiting rather than augmenting cell surface glycosylation (Goon & Bertozzi 2000). However, just as some of the glycosyltransferases are able to function with unnatural substrates, some metabolic pathways are able to accommodate small perturbations in the structure of their substrate, in particular the enzymes that synthesize sialic acid. Sialic acid is unusual in that it is the only nine-carbon sugar found on mammalian cells, which may account for the fact that the enzymes in the biosynthetic pathway are not stringently selective. The first committed step in sialic acid biosynthesis (Figure 7) is the epimerization of UDP-GlcNAc to *N*-acetylmannosamine (ManNAc) by UDP-GlcNAc/ManNAc-2-epimerase (Keppler et al. 1999). Cells can also take up exogenous ManNAc and utilize it in sialic acid biosynthesis. ManNAc from either source is phosphorylated by ManNAc-6-kinase and then condensed with PEP by sialic acid-9-P synthase. The phosphate is removed from the phosphorylated sialic acid by sialic acid-9-P phosphatase. CMP-sialic acid synthetase converts free sialic acid into CMP-sialic acid, which is transported to the Golgi. The sugar is then transferred from the sugar nucleotide onto a cell surface–bound protein by a Golgi resident sialyltransferase.

The importance of sialic acid as a terminal sugar in many oligosaccharides involved in cell adhesion and disease processes has already been discussed. Selectively altering the structure of sialic acids permits examination and manipulation of these sialic acid-mediated processes. This was first demonstrated by Reutter and coworkers using the one, two, and three carbon homologs of ManNAc shown in Figure 7; *N*-propanoylmannosamine (ManProp), *N*-butanoylmannosamine (ManBut), and *N*-pentanoylmannosamine (ManPent). These compounds were found to be converted into their respective sialic acids by living cells without affecting their growth rate or viability (Kayser et al. 1992). One would assume that a change in the size of the sialic acid side chain would affect binding of viruses that recognize sialic acid. However, how that effect would manifest itself is not immediately evident. Cells treated with ManProp, ManBut, and ManPent were exposed to two different polyoma viruses. One of the viruses showed dramatically increased binding, whereas binding of the other virus was reduced by as much as 86% (Keppler et al. 1995). Treatment of cells with the same three mannosamine derivatives resulted in inhibition of influenza A virus infection by as much as 80% (Keppler et al. 1998).

Variation of the sialic acid side chain evidently has profound biological consequences. This was also observed when fibroblast cells were cultured in the presence of ManProp, which resulted in suppression of contact-dependent growth inhibition (Wieser et al. 1996). The conversion of unnatural mannosamine analogs into unnatural cell surface sialic acids appears to be a universal feature of mammalian cells. ManProp was also shown to be metabolized by neural cells, which are the only cell type in adult humans that possesses sialic acid in polymer form (Schmidt et al. 1998). It was recently shown that substitution of a hydroxyl group on the

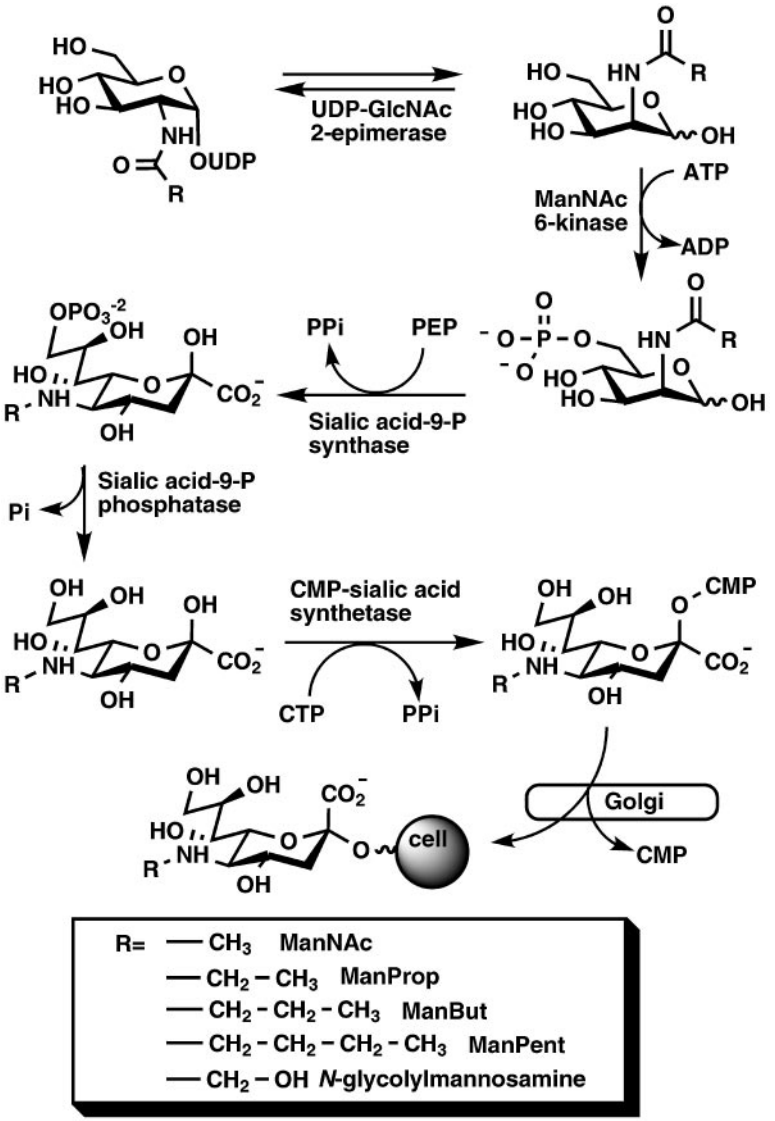

Figure 7 The sialic acid biosynthetic pathway.

N-acyl side chain is tolerated by the biosynthetic machinery, thereby producing *N*-glycolylsialic acid, an epitope that is otherwise not observed in human cells. This has a direct effect on binding of the myelin-associated glycoprotein and may lead to new methods for regeneration of damaged nerve tissue (Collins et al. 2000). Another feature of sialic acid is that it is frequently expressed at elevated levels in tumor antigens. Metabolism of mannosamine analogs into unnatural sialic acids on

tumor cells could sufficiently differentiate them from healthy cells, which would allow tumor cells to be selectively targeted by the immune system (Liu et al. 2000, Lemieux & Bertozzi 2001).

CHEMOSELECTIVE LIGATION TO METABOLICALLY INSTALLED UNNATURAL CELL SURFACE OLIGOSACCHARIDES

The utility of exogenous glycosyltransferases for cell surface modification is limited by the types of unnatural substrates the enzyme will accept. Metabolism of unnatural substrates is even more severely limited because it requires an entire enzyme pathway to be permissive for the substitution. The solution to this problem is to use metabolic engineering to install an unnatural cell surface sugar that can subsequently participate in a chemical reaction to add further functionality (Mahal & Bertozzi 1997). Figure 8*a* illustrates this concept. Cells are fed a carbohydrate bearing a uniquely reactive chemical handle (shown as a red asterisk) that is expressed on the cell surface. This chemical handle will react only when exposed to a reagent with carefully matched reactivity (the blue circle in Figure 8*a*). The reagent can be designed to bear any oligosaccharide or biological probe of interest (the blue oval in Figure 8*a*), which would result in its attachment to the cell surface. The challenge now shifts from engineering the carbohydrate substrate and enzymes to designing the appropriate reaction. Reactions that selectively form a covalent linkage in a biological medium are known as chemo-selective ligations (Lemieux & Bertozzi 1998).

Native sialic acid bears a unique chemical handle in the form of a terminal diol (Figure 8*b*). This functional group can be selectively oxidized using periodate to form an aldehyde that will then form a Schiff base in the presence of amines (Puri & Springer 1996). Schiff base formation is not a selective reaction given the preponderance of amines present at the cell surface. Ketones are electrophiles that are more stable than aldehydes and as such do not appreciably form Schiff bases in water. However, ketones do react selectively with *N*-acyl hydrazides under slightly acidic conditions (pH = 5–6.5) to form stable hydrazone linkages. Hydrazone formation is therefore chemo-selective in the context of the cell surface and is a means to construct conjugates of unlimited size and complexity.

In order to carry out hydrazone formation on the cell surface, the ketone handle must be incorporated into cell surface glycans. This was achieved via synthesis of a mannosamine analog called *N*-levulinoylmannosamine (ManLev; Figure 8*b*), with a ketone on the *N*-acyl sidechain. Treatment of cells with ManLev resulted in presentation of ketones on the cell surface. These ketones were quantified by reaction with biotin hydrazide, followed by labeling with fluorescein-derivatized avidin. In principle, any hydrazide reagent can be appended to cell surface glycans in this fashion (Mahal et al. 1997).

ManLev has the largest *N*-acyl group of any mannosamine analog that is successfully metabolized by cells. A panel of ketone-bearing mannosamine analogs with longer or bulkier side chains was synthesized and tested using the biotin hydrazide assay in order to determine the selectivity of sialic acid biosynthesis. None of these was appreciably converted to sialic acids. These potential substrates were acetylated in order to ensure that cell uptake was not the limiting step in their metabolism (Jacobs et al. 2000).

Use of the ketone for cell surface engineering is not limited to mannosamines and their metabolic product, sialic acid. Recent work has demonstrated that it is possible to install the ketone on an analog of a different sugar, *N*-acetylgalactosamine (GalNAc). The keto-GalNAc analog can be fed to cells without any observed detrimental effects and results in expression of cell surface ketones as detected by the biotin hydrazide assay. The analog synthesized in this case (Figure 8*c*) was not simply an extension of the *N*-acyl sidechain, but rather it is a ketone isostere where the nitrogen is replaced by carbon. Elimination of a potential hydrogen bond may be the reason that keto-GalNAc is best utilized by ldl-D cells, a mutant cell line unable to synthesize endogenous GalNAc (Hang & Bertozzi 2001). Such mutant cell lines could be used to express recombinant glycoproteins bearing the ketone handle on a large scale.

Applications of Ketone/Hydrazide Chemistry

Cell surface ketones in the form of SiaLev have been used to remodel the glycoconjugates of living cells. Sugars with aminooxy functionality (another group that reacts selectively with ketones) at the anomeric position can be ligated to the ketones, which results in a novel cell surface oligosaccharide (Yarema et al. 1998). The same reaction was used to target MRI contrast reagents to cells treated with ManLev. The contrast reagent was linked to an aminooxy group and shown to react selectively with cells that expressed ketones over cells that did not (Lemieux et al. 1999). A two-stage strategy was employed in order to engineer a receptor for viral-mediated gene therapy. Cell surface SiaLev was reacted with biotin hydrazide and then treated with an avidin-anti-adenovirus antibody conjugate. Selective attachment of the antibody via this method resulted in enhanced gene transfer to modified cells (Lee et al. 1999).

New Reaction for Cell Surface Modification: The Staudinger Ligation

The success of hydrazone formation with metabolically introduced cell surface ketones prompted the search for an alternative cell surface ligation strategy. The ketone is relatively sterically demanding and therefore may not be appropriate for investigation of other pathways less forgiving than sialic acid. In addition, there are applications that may benefit from tandem cell surface ligations, such as installation of two different cell surface receptors or dyes. The cell surface Staudinger

ligation utilizes *N*-azidoacetylmannosamine (ManAzide; Figure 9) that is metabolically converted into SiaAzide. The azidoacetate side chain is somewhat smaller than the levulinoyl group and therefore may be more readily incorporated. Once on the cell surface the azide is available for reaction with a phosphine bearing the biological moiety of interest. The reaction takes place cleanly at room temperature and pH 7.4, resulting in the formation of a stable amide bond (Saxon & Bertozzi 2000).

CONCLUSION

The manipulation of cell surface glycosylation patterns can now be achieved by several methods. Genome-sequencing projects have presented a complete repertoire of biosynthetic enzymes, and selective inhibition of these with small molecules can disrupt the cell surface expression of specific epitopes. Moreover, the increasing availability of structural information for the glycosyltransferase family may enable opportunities in enzyme engineering. Altered glycosyltransferases may be designed to transfer unnatural monosaccharides or higher order structures onto cells for biological studies. Advances in automated oligosaccharide synthesis are important in this regard because complex structures can now be obtained by relative novices and exploited in cell surface studies.

Metabolic approaches to modifying cell surface glycans are still in their infancy, but significant progress has already been achieved using the sialic acid pathway. Replacement of cell surface sugars with slightly altered variants is analogous to site-directed mutagenesis. Unlike enzyme inhibition or gene knockouts, which delete entire monosaccharides from cell surface glycans, metabolic substitution can alter these structures in a more subtle manner, thereby allowing detailed structure/activity correlations to be performed in the context of a cell surface. The generality of this approach to other biosynthetic pathways has yet to be determined.

Finally, the convergence of metabolic engineering with chemical modification allows one to exercise considerable creativity in remodeling cell surfaces. The method may be extended to the reconstruction of complex cell surface architectures using large synthetic macromolecules (proteins and glycoproteins) as substrates. Future studies of glycobiology will benefit from the combined application of chemical, biochemical, and genetic techniques.

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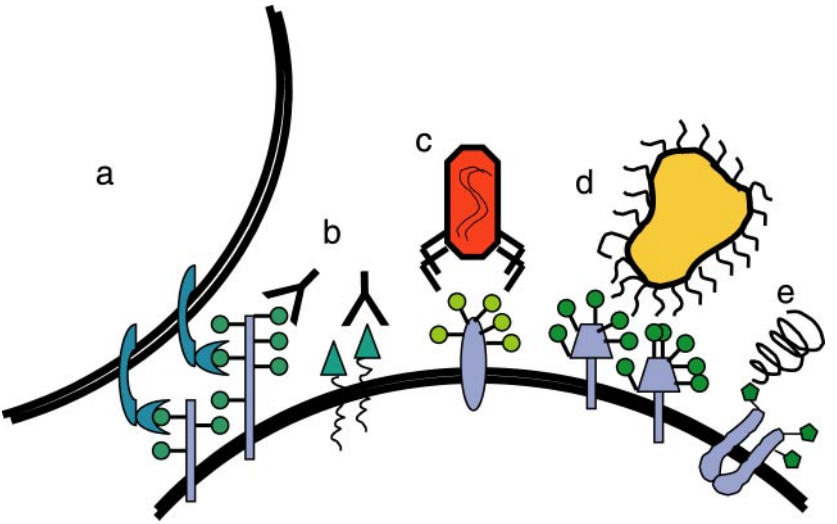

Figure 1 Cell surface carbohydrates mediate interactions between a cell and (*a*) another cell, (*b*) antibodies, (*c*) viruses, (*d*) bacteria, and (*e*) peptide hormones or toxins.

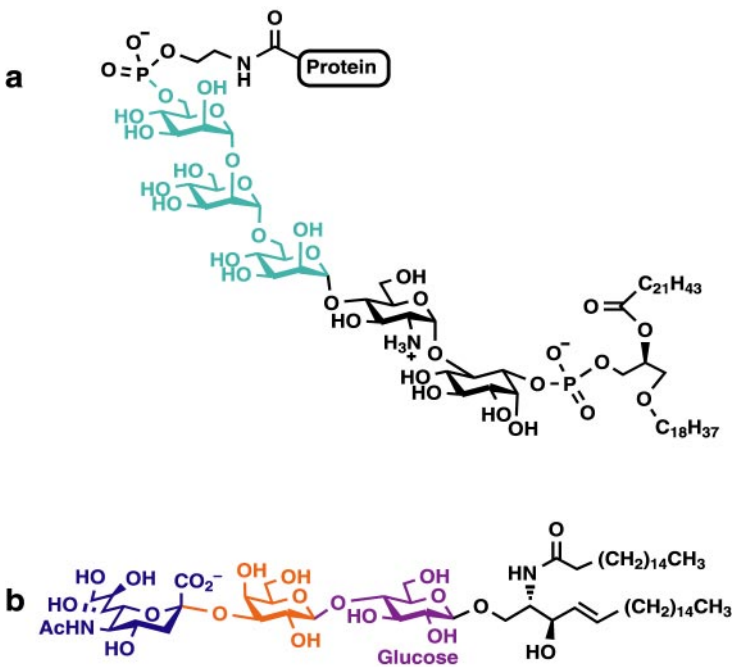

Figure 3 Examples of two of the five types of glycoconjugates with structures of common cell surface monosaccharides highlighted: (*a*) structure of a GPI anchor and (*b*) the ganglioside GM_3 an example of a glycolipid.

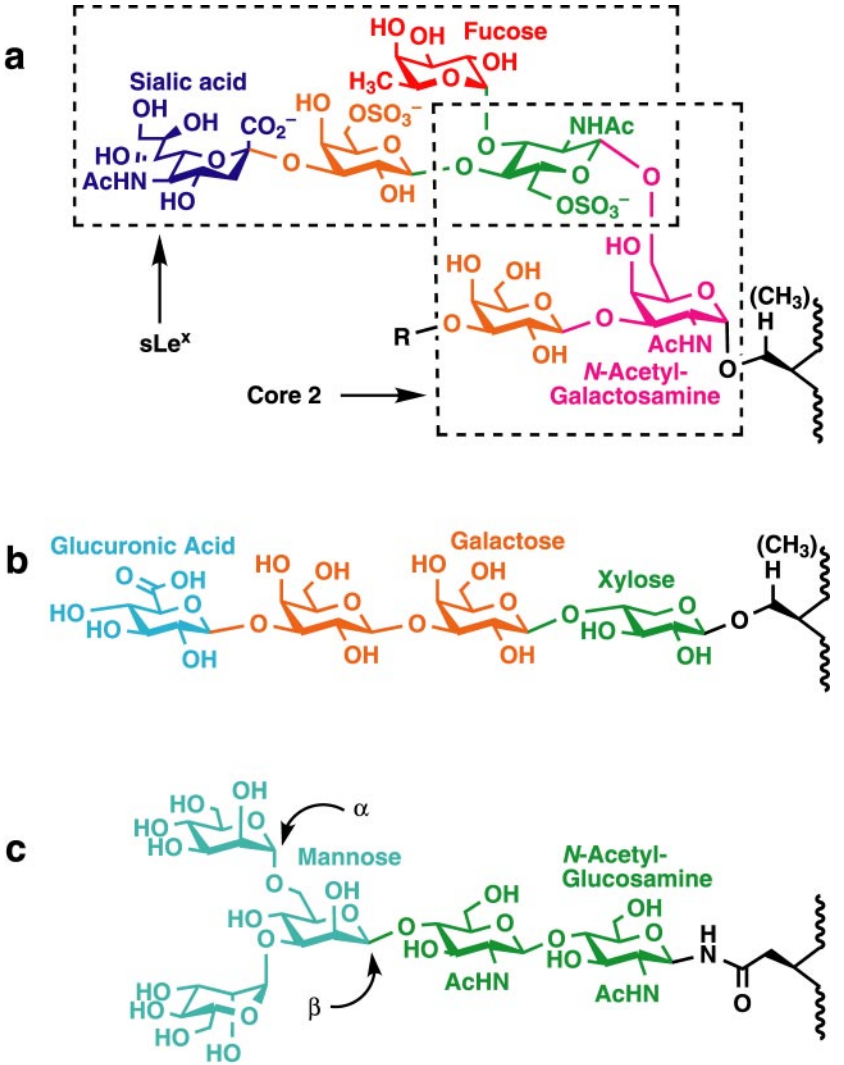

Figure 2 Examples of three of the five types of glycoconjugates with structures of common cell surface monosaccharides highlighted: (*a*) an *O*-linked glycan bearing the sulfated sLe^x tetrasaccharide, (*b*) the core region of a glycosaminoglycan, and (*c*) the core region of an *N*-linked glycan.

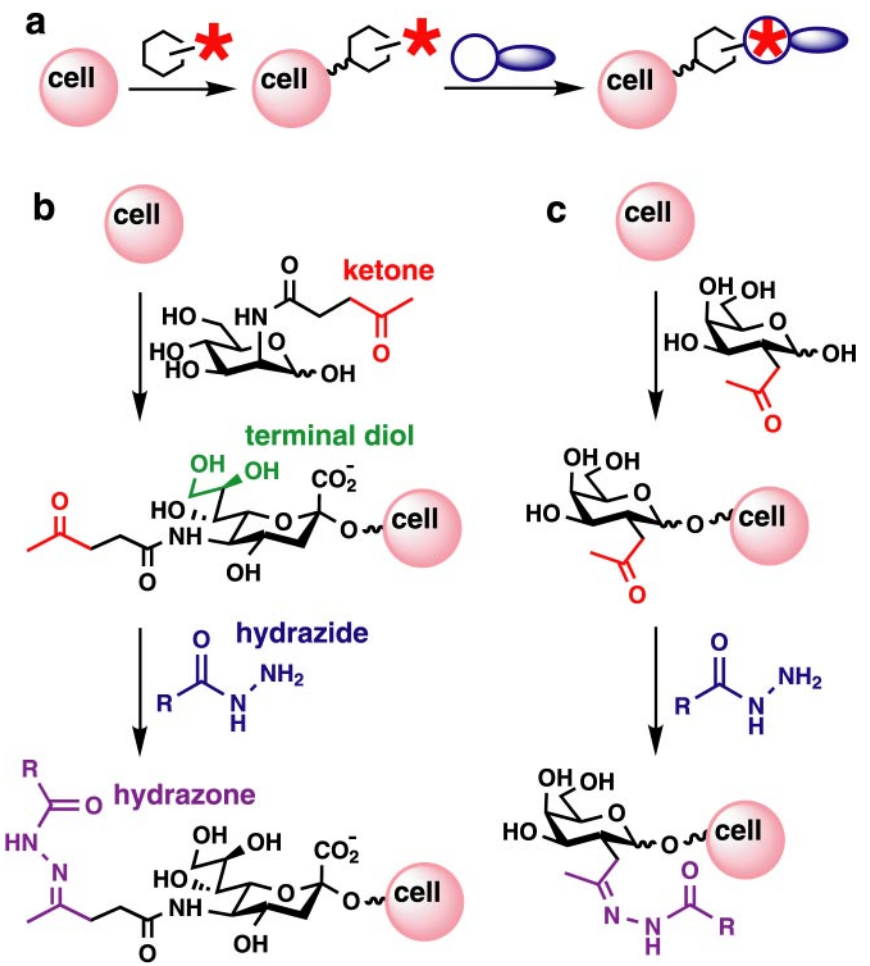

Figure 8 (*a*) Metabolism of a sugar bearing a reactive functional group results in a cell surface display of that functional group. Chemical reaction with an appropriate reaction partner remodels the cell surface. (*b*) ManLev delivers ketones to the cell surface that react with hydrazides to form stable hydrazones. (*c*) Keto-GalNAc delivers ketones to the cell surface in ldl-D cells.

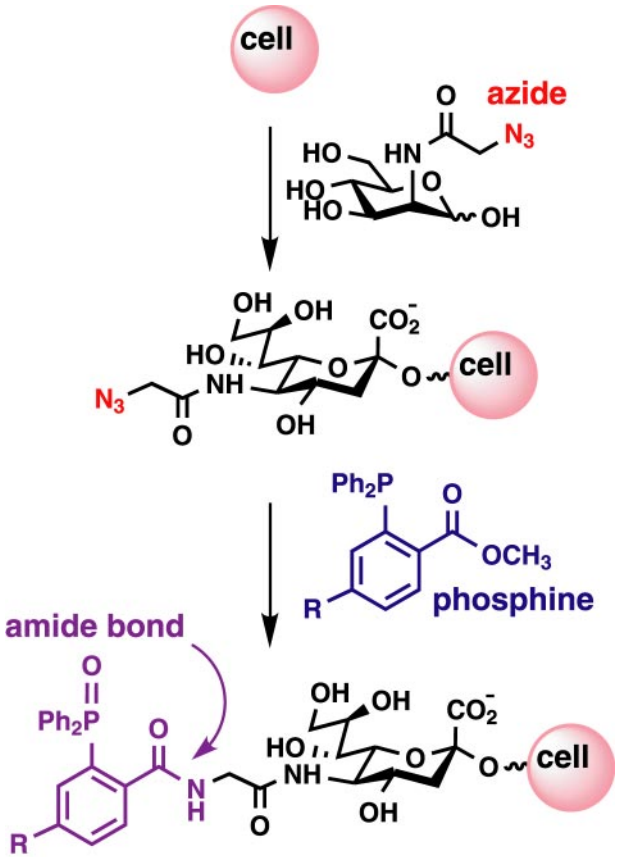

Figure 9 A new reaction for cell surface modification. Metabolism of ManAzide results in display of cell surface azides. Reaction with exogenously delivered phosphine via the Staudinger ligation creates an amide-linked cell surface conjugate.